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Chromosomal copy number changes of locally advanced rectal cancers treated with preoperative chemoradiotherapy

Marian Grade[a,b,1], Jochen Gaedcke[a,1], Danny Wangsa[b], Sudhir Varma[c], Jaje Beckmann[a], Torsten Liersch[a], Clemens Hess[d], Heinz Becker[a], Michael J. Difilippantonio[b], Thomas Ried[b,*], B. Michael Ghadimi[a,*]

[a]*Department of General and Visceral Surgery, University Medicine, Georg-August-University, Robert Koch Str. 40, 37075 Göttingen, Germany*
[b]*Genetics Branch, National Cancer Institute, National Institutes of Health, 50 South Drive, Bethesda, MD 20892-8010*
[c]*Biometrics Research Branch, National Cancer Institute, National Institutes of Health, 6130 Executive Plaza, Rockville, MD 20852*
[d]*Department of Radiation Oncology and Radiotherapy, University Medicine, Georg-August-University, Robert Koch Str. 40, 37075 Göttingen, Germany*

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Abstract Standard treatment of rectal cancer patients comprises preoperative chemoradiotherapy followed by radical surgery. However, clinicians are faced with the problem that response rates vary from one individual to another. Predictive biomarkers would therefore be helpful. To identify genomic imbalances that might assist in stratifying tumors into responsive or nonresponsive categories, we used metaphase comparative genomic hybridization to prospectively analyze pretherapeutic biopsies from 42 patients with locally advanced rectal cancers. These patients were subsequently treated with 5-fluorouracil–based preoperative chemoradiotherapy. Based on downsizing of the T-category, 21 rectal cancers were later classified as responsive, while the other 21 were nonresponsive. Comparing these two groups, we could show that gains of chromosomal regions 7q32~q36 and 7q11~q31, as well as amplifications of 20q11~q13, were significantly associated with responsiveness to preoperative chemoradiotherapy ($P < 0.05$). However, the probability of detecting these copy number changes by chance is high ($P = 0.21$). Our primary results suggest that pretherapeutic evaluation of chromosomal copy number changes may be of value for response prediction of rectal cancers to preoperative chemoradiotherapy. This will require validation in a larger cohort of patients.

1. Introduction

According to the results of the CAO/ARO/AIO-94 trial of the German Rectal Cancer Study Group, preoperative 5-FU–based chemoradiotherapy (CT/RT) is recommended for locally advanced rectal cancers (UICC stage II/III) in Germany, large parts of Europe, and the United States [1]. Clincians, however, face a considerable problem because the response of individual tumors to preoperative CT/RT is very heterogeneous, ranging from complete response to resistance. As a result, phase-I/II trials have been initiated to explore whether intensifying preoperative treatment could increase the rate of complete tumor remission, which has been demonstrated to result in a pronounced survival benefit [2], and to reduce the risk of metastatic spread [3–6].

Regardless of these improvements, it obviously remains of considerable clinical interest to identify pretherapeutic markers of response. In a previous investigation, we were able to identify a set of 54 genes that were differentially expressed in a significant manner between responsive and nonresponsive tumors [7]. We could subsequently show that these gene expression signatures also correlated with an increased risk of cancer recurrence [8]. Since such analyses have not yet been conducted on the DNA level, we wished to explore whether significant differences can also be detected in the tumor genomes using metaphase comparative genomic hybridization (CGH).

2. Materials and methods

2.1. Selection of patients, study design, and treatment

All 42 patients participated in the CAO/ARO/AIO-94 [1] or CAO/ARO/AIO-04 trial of the German Rectal Cancer

* Corresponding authors. Tel.: +49-551-398730; fax: +49-551-3991315 or Tel.: 301-594-3118; fax: 301-435-4428.
E-mail address: riedt@mail.nih.gov (T. Ried) or mghadim@uni-goettingen.de (B.M. Ghadimi).
[1] Both authors contributed equally to this work.

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Study Group, and were treated at the Department of General and Visceral Surgery, University Medicine Göttingen (Göttingen, Germany). Preoperative CT/RT, surgical resection, and pathologic workup were standardized according to the guidelines of these randomized phase-III trials.

Pretherapeutic staging included rigid rectoscopy and endorectal ultrasound, colonoscopy, abdominal and pelvic computed tomography, and chest x-ray. Only locally advanced adenocarcinomas (cUICC II/III) located within 12 cm from the anocutaneous verge were included. All patients subsequently received a total radiation dose of 50.4 Gy (single dose of 1.8 Gy) accompanied by a 120-hour continuous intravenous application of 5-FU (1,000 mg/m^2/day on days 1–5 and days 28–33). After an interval of approximately 6 weeks after completion of CT/RT, standardized surgery was performed, which included total mesorectal excision [9]. The clinical data are summarized in Table 1, and the experimental design is illustrated in Fig. 1.

2.2. Ascertainment of tumor biopsies

From each patient, we prospectively collected pretherapeutic biopsies from adjacent representative areas of the tumors, adhering to the guidelines set by the local ethical

Table 1
Clinical data of 42 patients

Tumor samples	Sex	Age	uT	ypT	Response	uN	ypN	ypN total	ypN infiltrated	ypGrading	R	cIUCC	ypUICC
P1	M	61	3	0	+	0	0	18	0	x	0	II	0
P2	M	61	3	0	+	1	0	27	0	x	0	III	0
P4	M	68	3	2	+	1	0	22	0	2	0	III	I
P6	M	65	3	2	+	1	0	24	0	2	0	III	I
P7	M	49	3	1	+	0	0	18	0	2	0	II	II
P10	M	53	3	3b	–	1	1	30	1	2	0	III	III
P11	M	64	3	3b	–	1	0	15	0	2	0	III	II
P12	M	55	3	3b	–	0	0	8	1	3	0	II	III
P13	F	70	3	3b	–	1	1	27	1	2	0	III	III
P14	M	58	3	4a	–	1	1	19	1	2	0	III	III
P15	M	53	3	3b	–	1	0	28	0	2	0	III	II
P17	M	78	3	3b	–	1	1	19	2	3	0	III	III
P20	M	58	3	3c	–	0	0	16	0	2	0	II	II
P21	F	59	3	3c	–	1	0	17	0	2	0	III	II
P22	M	62	3	3a	–	1	0	14	0	2	0	III	II
P23	F	40	3	4a	–	1	1	22	1	2	0	III	III
P24	M	68	3	2	+	0	0	16	0	2	0	II	I
P26	M	62	3	3	–	1	0	20	0	2	0	III	II
P28	M	59	3	Tis	+	1	1	17	1	3	0	III	III
P29	F	68	3	3	–	1	0	15	0	2	0	III	II
P30	M	71	3	2	+	0	0	12	0	2	0	II	I
P31	M	63	3	1	+	1	0	24	0	2	0	III	I
P32	M	50	3	3d	–	0	0	34	0	2	0	II	II
P33	F	58	3	3a	–	0	0	12	0	2	0	II	II
P34	M	68	3	2	+	1	0	12	0	2	0	III	I
P35	M	62	3	3	–	0	0	26	0	2\3	0	II	II
P36	M	66	3	0	+	1	0	19	0	X	0	III	0
P37	M	61	3	4	–	1	2	22	6	2	0	III	III
P38	M	57	3	2	+	0	0	24	0	2	0	II	I
P39	M	70	4	3c	+	1	2	25	5	2	0	III	III
P40	M	73	3	3a	–	1	1	31	1	2	0	III	III
P41	M	59	3	2	+	1	0	5	0	2	0	III	I
P42	M	64	3	1	+	1	0	37	0	1\2	0	III	I
P43	F	48	3	1	+	1	0	47	0	1\2	0	III	I
P44	M	50	3	3b	–	0	0	26	0	2\3	0	II	II
P45	M	70	3	2	+	0	0	22	0	2\3	0	II	I
P46	F	71	3	2	+	0	0	30	0	2	0	II	I
P47	F	67	3	3b	–	0	1	15	2	2	0	II	III
P48	M	52	3	2	+	1	0	18	0	2	1	III	II
P49	M	70	3	3c	–	1	1	37	1	3	0	III	III
P50	F	53	3	1	+	1	0	20	0	2	0	III	I
P51	M	65	3	0	+	0	0	30	0	X	0	II	0

Abbreviations: uT, pretherapeutic T category determined by endorectal ultrasound; ypT, T category determined by histopathological assessment after preoperative chemoradiotherapy; uN, lymph node status by endorectal ultrasound; ypN, lymph node status by histopathologic assessment; ypN total, total number of analyzed lymph nodes; ypN infiltrated, number of infiltrated lymph nodes; ypGrading, tumor grading by histopathologic assessment; R, resectability (surgical resection margins); cUICC, clinical UICC stage; ypUICC, post-treatment UICC stage; UICC, International Union Against Cancer; Tis, tumor in situ.

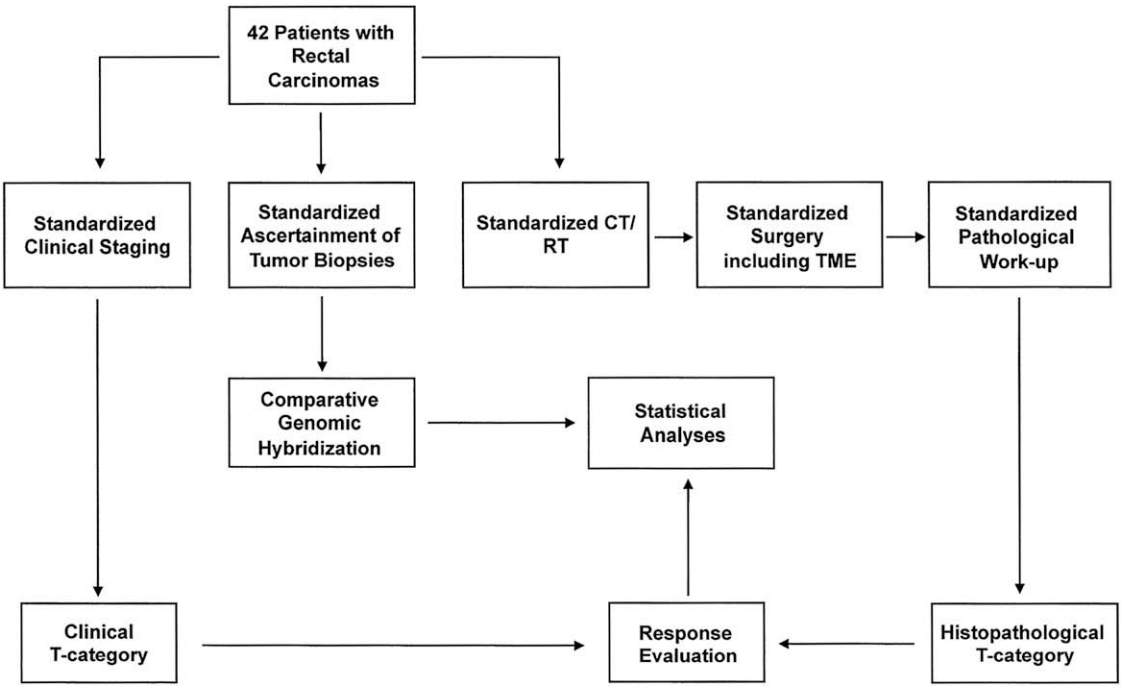

Fig. 1. Pictorial presentation of study design. CT/RT, chemoradiotherapy; TME, total mesorectal excision.

review board. The first one was used for histopathologic confirmation of tumor diagnosis, and the second one was immediately stored in RNAlater (Ambion, Austin, TX) for subsequent extraction of nucleic acids.

2.3. Classification of response

Response was defined as downsizing of the primary tumor by comparing the pretherapeutic T-category (determined by endorectal ultrasound) with the histopathologic T-category (after surgical resection). As described previously, tumors exhibiting a T-level downsizing of at least one level were considered responsive [7,8].

2.4. Isolation of tumor DNA and CGH

DNA was isolated using TRIZOL (Invitrogen, Carlsbad, CA) following standard procedures, and comparative genomic hybridization was performed as described previously [10]. The protocol can be found at http://www.riedlab.nci.nih.gov/protocols.asp. Briefly, 200 ng of tumor and sex-matched normal genomic DNA, nick translation–labeled with biotin-16-dUTP (Roche, Mannheim, Germany) or digoxigenin-12-dUTP (Roche), respectively, were combined with an excess (20 μg) of the Cot-1 fraction of human DNA (Invitrogen, Carlsbad, CA) and precipitated. DNA was resuspended in a hybridization solution (50% formamide, 2× standard saline citrate, 10% dextran sulfate), denatured, pre-annealed for 1 hour at 37°C, and applied to pretreated and denatured slides containing normal human metaphase spreads. Hybridization was performed at 37°C in a moist chamber for 72 hours. After post-hybridization washes, tumor DNA was detected with Avidin-FITC (Vector, Burlingame, CA), and the reference DNA was detected with mouse anti-digoxigenin (Sigma, St. Louis, MO). The slides were counterstained with 4'-6,diamidino-2-phenylindole and embedded in an antifade solution containing para-phenylene-diamine (Sigma).

Images were acquired for each fluorochrome using a cooled CCD camera (DFC 350 FX; Leica, Bensheim, Germany) coupled to an epifluorescence microscope (DM 6000; Leica) containing fluorochrome-specific filter sets. For automated karyotyping and analysis, CW-4000 imaging software (Leica, Cambridge, UK) was used.

2.5. Statistical analysis: chromosomal imbalances and clinical response to preoperative chemoradiotherapy

To identify chromosomal loci that were differentially affected by copy number changes in responsive and nonresponsive tumors, we first divided the human genome into 320 bands according to the cytogenetic regions of the International System of Cytogenetic Nomenclature [11]. The p-arms of the acrocentric chromosomes 13, 14, 15, 21, and 22, as well as the centromeres and the entire X and Y chromosome, were excluded from further analyses, leaving a final set of 260 chromosome bands. For each band, we assigned a numerical value corresponding to a chromosomal loss (−1), no chromosomal change (0), chromosomal gain (+1), or amplification (+2). Clustering of those bands that exhibited exactly the same

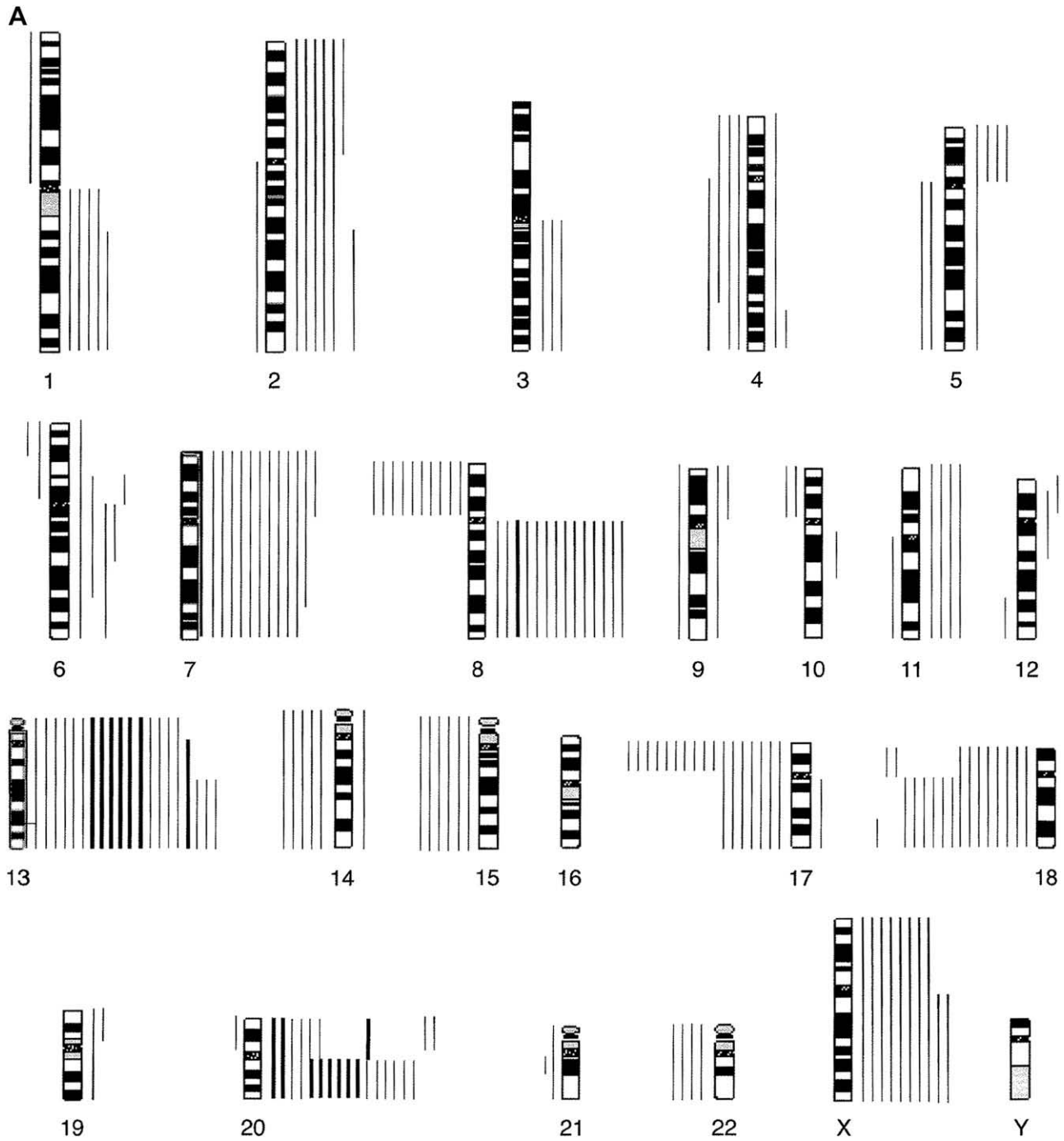

Fig. 2. CGH profiles of 21 responsive (A) and 21 nonresponsive rectal cancers (B) Genomic copy number losses are indicated as bars to the left of the chromosome, while bars to the right of the chromosome represent copy number gains. Thick bars indicate chromosomal amplifications.

patterns of gains or losses (i.e., linkage) in the tumor samples resulted in 69 band groups.

To identify chromosomal imbalances associated with response to CT/RT, we applied the Wilcoxon statistic. As a rank statistic, it arranges observations in ascending order and uses their rank instead of the actual observation value. These ranks are combined in a rank statistic, which forms the basis for further analysis of the difference between the medians of the two groups. We used a permutation method for computing the *P* value for the rank statistic in lieu of the classic method because the latter becomes problematic when there are a large number of ties between the observations. To compute the CGH *P* value for each band group, we repeatedly permuted the class labels (response and nonresponse indicators) and calculated the proportion of times the rank-statistic of the resulting data set was more extreme than the one we obtained.

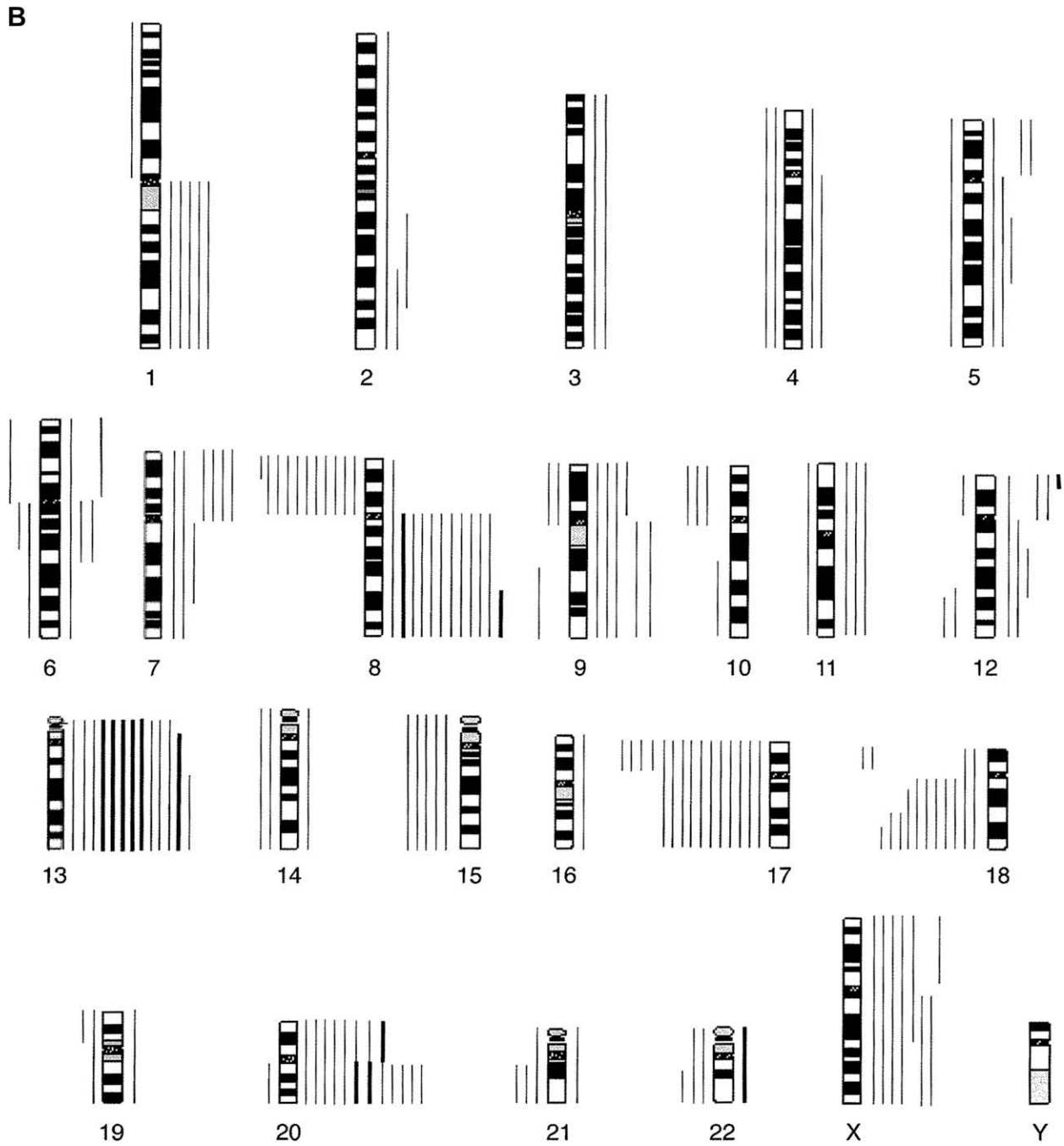

Fig. 2. (Continued).

3. Results

We have previously reported that a set of 54 differentially expressed genes allows response prediction of rectal cancers to CT/RT with an accuracy of 83% [7], and, very recently, we were able to show that these gene expression signatures correlated with the risk of developing recurrent disease [8]. We also previously reported a linear relationship of genomic copy number with average gene expression levels [10,12,13]. Now we aimed to examine whether the mechanism of transcriptional deregulation of specific genes involved in response prediction relates to genomic copy number variations as well.

3.1. Patient characteristics

Forty-one patients were diagnosed with uT3 carcinomas, while one patient exhibited a uT4 carcinoma. Response of the 42 rectal adenocarcinomas (cUICC II, $n = 15$ and cUICC III, $n = 27$) to preoperative CT/RT, based on T-level

downsizing, resulted in the classification of 21 prospectively collected biopsies as responders (P1, P2, P4, P6, P7, P24, P28, P30, P31, P34, P36, P38, P39, P41–43, P45, P46, P48, P50, and P51), and the remaining 21 patients as nonresponders (P10–15, P17, P20–23, P26, P29, P32, P33, P35, P37, P40, P44, P47, and P49; Table 1).

3.2. Chromosomal imbalances

To identify potential differences in the patterns of chromosomal gains and losses in responsive and nonresponsive tumors, we analyzed all cases with CGH. The results of the individual CGH experiments are depicted in Table 2 and Fig. 2 (A and B). Within these karyograms, lines to the left of the chromosomal ideograms indicate chromosomal losses (ratio of 0.8), and lines to the right depict chromosomal gains (ratio of 1.2). Amplifications (ratio of > 1.5) are drawn as bold lines.

Copy number gains most frequently affected chromosome arms 7p (40%), 8q (52%), 13q (67%), 20p (38%), and 20q (67%), while frequent losses mapped to chromosome arms 8p (45%), 17p (74%), and 18q (43%). These findings are in concordance with previous reports on colorectal carcinomas [10,12–17]. For a detailed case summary, see http://www.ncbi.nlm.nih.gov/sky/skyweb.cgi. While only one case (P37) did not display any genomic imbalances, the remaining 41 tumors were aneuploid, with aberrations affecting between 1 and 18 chromosomes. Dividing the total number of chromosomal copy alterations ($n = 330$) by the number of tumors analyzed ($n = 42$), we obtained an average number of copy alterations value of 7.9 [18]. Amplifications were mapped to chromosome arms

Table 2
Chromosomal gains and losses of 42 locally advanced rectal cancers. The suffix ++ indicates chromosomal amplification

Tumor Samples	Chromosomal gains	Chromosomal losses
P1	5p, 7p, 8q, 13q21~ter, 20p, Xq	6p22~pter, 10p, 15, 17, 18p
P2	13, 20q	14, 17p, 18q
P4	20q	–
P6	7, 13, 20q++	4, 8p, 18q
P7	1q, 3q, 7p, 7q11~q31, 13++, 20p, 20q++	8p, 11q, 14, 15, 18
P10	2q32~ter, 8q23-ter++, 9, 20p, 20q++	4, 5, 6q, 8p, 15, 17, 18q
P11	8q, 13	6p, 8p, 9q22~qter, 10p, 15, 17, 20, 21
P12	–	17
P13	4	17, 20q
P14	8q, 13	10p, 12p, 12q23~qter, 17
P15	1q, 7p, 9q, 13, 16, 19, 20	4, 18q
P17	9q, 20q	9p
P20	5p, 8q, 13, 20	8p, 9p, 10p, 18q22~qter, 19p
P21	7, 8q, 13, 20q	8p, 12q24.3~qter, 14, 15, 17p, 18p, 21q22
P22	1q, 2, 3, 5, 6p, 8, 12, 13++, 14, 20p++, 20q, 21, Xp	17, 18p
P23	1q, 4q, 9, 12p13++, 13++, 20, Xq	1p, 6q11~q16, 11, 14, 17, 18
P24	6p11~p21.3, 8q, 13, 17q, 20q++	17p
P26	7p, 13, Xp, Xq11~q21	17p, 18q
P28	7, 13++, 20q++	4q, 9, 10p, 14, 15, 18, 20p
P29	–	17
P30	1q, 2, 3q, 6, 7, 8q, 9, 11, 12p12-12q14, 13++, 14, 20, X	8p, 12q23~qter, 15, 17, 18
P31	2, 5p, 6q, 7, 8q, 11, 13++, 20p, 20q++, X	5q, 8p, 17p, 18
P32	2q22~q34, 8q, 11, 13++, 22++, X	8p, 17, 18q21~qter
P33	3, 5p, 6, 7p, 8q, 9, 11, 12q14~q22, 13++, 20p, 20q++, Xq	8p, 17p, 18, 19, 22
P34	6q11~q22, 8q, 13, X	17, 18p, 22
P35	8q, 20	8p, 15, 18q
P36	1q, 2p, 4q32~ter, 7, 12p, 13	1p, 2q, 4p, 4q11~q31.3, 17p, 18, 22
P37	–	–
P38	2q23~q33, 6p12~q23, 8q, 10q21~q22, 11, 13	17
P39	2, 8q, 11, 13++, 20++, X	8p, 17, 18q
P40	5q14~q23, 6q11~q22, 7p, 8q++, 9p, 11, 12p, 13++, 20, X	8p, 15, 17p, 18q, 22
P41	4, 5, 8q, 13q21~qter, 20p++, 20q, X	6p, 14, 17, 18q, 22
P42	2, 8q, 13q21~qter, 20p, Xq	8p, 17p, 21
P43	7, 8q, 13, 20q	8p, 17p, 18, 21q22
P44	6q11~q22, 7, 8q, 13q21~qter, 20q, X	8p, 17, 18q12~qter, 21q22, 22q12~qter
P45	5p, 7, 8q, 13++, 19p, 20++, X	5q, 15, 17, 18q
P46	2, 7, 8q++, 13, 20q++	8p, 17p, 18, 22
P47	1q, 8q, 12p	8p, 10q22~qter, 17
P48	13, 20q, X	17p, 18q22~qter
P49	1q, 5q, 7q11~q31, 12q, 13q++, 20q, X	8p21~pter, 18q21~qter
P50	1q, 7, 8q, 13q++, 19, 20q	4, 8p, 15, 17p, 18
P51	1q22~qter, 3q, 7, 8q, 9p, 13, 20, X	8p, 14, 17p, 18q

8q ($n = 2$), 13q ($n = 12$), 20p ($n = 2$), and 20q ($n = 8$), as well as chromosomes 20 ($n = 2$) and 22 ($n = 1$). Regional amplifications were located on chromosomes 8q23~ter and 12p13.

3.3. Chromosomal imbalances and clinical response to preoperative chemoradiotherapy

Comparing the chromosomal imbalances of 21 responders and 21 nonresponders, we observed that the majority of copy number changes were present at higher frequencies in the responsive tumors (Fig. 2, A and B). To achieve a more objective measure of the genomic instability, we calculated the average number of copy alterations. The average number of copy alterations values were 8.9 for the responders and 6.8 for the nonresponders.

Using the permutation P values for the rank-statistic, three different band groups were identified, which were significantly different ($P < 0.05$): 7q32~7q36 ($P = 0.015$), 7q11~7q31 ($P = 0.025$), and 20q11~20q13 ($P = 0.04$; Fig. 3). To account for multiple testing, we calculated the probability of obtaining three band groups with $P < 0.05$ by chance. This was done by permuting the class labels (thus removing any correlation between response and gain/loss), again calculating CGH P values, and estimating the proportion of times three or more band groups with a $P < 0.05$ were obtained. The corresponding P value was determined to be $P = 0.21$. Thus, we cannot reject the possibility that these three chromosomal aberrations were discovered by random chance. We were then curious to explore whether those tumors that showed a complete regression after preoperative CT/RT exhibited specific DNA aberrations. However, we could not detect significant differences between these tumors and those with a partial regression or complete remission (data not shown).

3.4. Correlation of chromosomal imbalances and gene expression signatures

To find further evidence that these three chromosome bands differentiate responsive and nonresponsive tumors, we used previously established gene expression signatures for 12/42 tumors [7] to calculate a measure of the overall differential expression of the genes belonging to a particular band group (the gene expression P value). The LS statistic from the Gene Set Class Comparison tool of BRB ArrayTools was used for these analyses [19]. The LS statistic reflects the mean of the negative logarithms of the individual P values for differential expression for all the genes present in the band group [20]. A large number of genes with moderately small P for differential expression will result in a large value for this statistic, as will a small number of genes with very small P values. Thus, the LS statistic captures the overall correlations between the expressions of the genes present in the band groups for response/nonresponse. A P value for the LS statistic is derived by randomly selecting k genes from the array and then computing the LS statistic for this group. The proportion of times an equal or higher LS statistic is obtained using this procedure is an estimate of the P value, which we defined as the ‘‘gene expression P value.’’

However, insignificant gene expression P values were obtained for all three band groups, i.e., $P = 0.26$ (7q32~7q36), $P = 0.054$ (7q11~7q31), and $P = 0.77$ (20q11~20q13). Thus, the response status is not influenced by altered expression of the genes residing on those chromosomal regions that differentiate responders from nonresponders (data not shown).

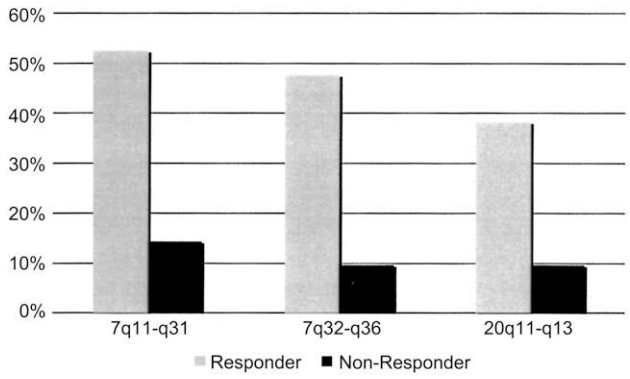

Fig. 3. Frequency of distinct subchromosomal alterations comparing responsive and nonresponsive tumors.

4. Discussion

Standard treatment of locally advanced rectal adenocarcinomas includes preoperative CT/RT followed by radical surgery [1]. However, clinical responsiveness to multimodal therapy strategies ranges from complete response to resistance. A pretherapeutic stratification of cancer patients into responders (who would benefit from standard 5-FU based CT/RT) and nonresponders (who might benefit from more aggressive or alternative therapies) therefore remains of high clinical value for individualized therapy planning.

Numerous immunohistochemical studies have been conducted to predict response to preoperative CT/RT. The most frequently analyzed proteins were p53 [21–36], p27 [28,37], thymidylate synthetase [29,35,38,39], bcl-2 [23,25,26,29,30,32,34], Ki-67 [23,30,32,34,36], and PCNA [23,33,40], but the results remain contradictory.

Unfortunately, differences in the clinical evaluation of the patients in these studies make it extremely cumbersome to dissect the cause of the conflicting results. First, different definitions of response were used (i.e., T-level downsizing, reduction of the tumor diameter or tumor volume, and histomorphologic regression grading). Second, tumor staging was performed with different diagnostic methods (i.e., magnetic resonance imaging, computed tomography, endorectal ultrasound, or clinical assessment). Third, therapeutic strategies varied dramatically; i.e., some clinics used radiation alone, and some applied chemoradiotherapy

with 5-FU monotherapy or 5-FU combined with oxaliplatin. Other investigators even added hyperthermia.

We had previously investigated whether there exist pretherapeutic gene expression signatures that characterize the clinical response of rectal adenocarcinomas to preoperative CT/RT [7]. Analyzing 30 biopsies using expression microarrays, we identified a set of 54 genes that showed significantly different ($P < 0.001$) expression levels between responders and nonresponders. These genes have been recently shown to correlate with the development of metastatic disease in these patients [8]. In the present study, we wished to explore whether differences between responsive and nonresponsive tumors can also be observed on the DNA level. We therefore screened pretherapeutic biopsies from 42 patients with locally advanced rectal cancers using chromosome CGH. All patients participated in prospective phase-III clinical trials and received 5-FU–based preoperative CT/RT. To the best of our knowledge, this is the first study to systematically correlate copy number profiles of rectal carcinomas with response to preoperative treatment.

We first observed that the identified chromosomal imbalances are in concordance with previous reports on colorectal carcinomas (recently reviewed in Grade et al. [41]). It is of interest to note that responsive tumors revealed a higher frequency of chromosomal copy number changes, which is reflected by a higher ANCA value (8.9 compared to 6.8). When we performed a Wilcoxon rank statistic, we obtained three band groups that were significantly differentially gained/amplified between responsive and nonresponsive tumors (Fig. 3): 7q32~7q36 ($P = 0.015$), 7q11~7q31 ($P = 0.025$), and 20q11~20q13 ($P = 0.040$). However, we also calculated a P value of 0.21 for the likelihood that these aberrations were identified by chance.

To find further evidence that these three chromosomal bands represent differentiating characteristics between responsive and nonresponsive tumors, we used previously obtained gene expression signatures for 12 of these 42 tumors to investigate whether these chromosomal alterations influence the clinical response by altering the expression of its resident genes. We specifically focused on these 12 tumors because corresponding gene expression profiles were available only for these patients. However, we again obtained insignificant gene expression P values for all three band groups [i.e., $P = 0.26$ (7q32~7q36), $P = 0.054$ (7q11~7q31), and $P = 0.77$ (20q11~20q13)], which means that responsive and nonresponsive tumors show similar expression values for the genes residing on these three chromosomal regions.

In summary, we identified three chromosomal regions that exhibited different copy numbers comparing tumors that were responsive and nonresponsive to preoperative chemoradiotherapy. However, there remains the possibility that these genomic copy number changes do not represent true biologic differences between responsive and nonresponsive tumors — just artificial noise. One may speculate that the relatively small sample size precluded significant results. We believe this to be unlikely because the response prediction of rectal carcinomas to preoperative chemoradiotherapy has already been performed successfully using gene expression microarrays with smaller or similar data sets [7,42,43]. However, the data are promising enough that we plan to use high-resolution array CGH to repeat mapping of chromosomal imbalances. Integrated into a Clinical Research Unit entitled "Biological Basis of Individual Tumor Response in Patients with Rectal Cancer" (http://www.kfo179.de), we have therefore initiated such an analysis.

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